

MSG5, a novel protein phosphatase promotes adaptation to pheromone response in *S.cerevisiae*

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Pheromone-stimulated yeast cells and haploid *gpa1* deletion mutants arrest their cell cycle in G₁. Overexpression of a novel gene called *MSG5* suppresses this inhibition of cell division. Loss of *MSG5* function leads to a diminished adaptive response to pheromone. Genetic analysis indicates that *MSG5* acts at a stage where the protein kinases *STE7* and *FUS3* function to transmit the pheromone-induced signal. Since loss of *MSG5* function causes an increase in *FUS3* enzyme activity but not *STE7* activity, we propose that *MSG5* impinges on the pathway at *FUS3*. Sequence analysis suggests that *MSG5* encodes a protein tyrosine phosphatase. This is supported by the finding that recombinant *MSG5* has phosphatase activity *in vitro* and is able to inactivate autophosphorylated *FUS3*. Thus *MSG5* might stimulate recovery from pheromone by regulating the phosphorylation state of *FUS3*.

Key words: MAP kinase/*MSG5*/pheromone response/protein phosphatases/*S.cerevisiae*

Introduction

The yeast *Saccharomyces cerevisiae* has two haploid cell types, *a* and α , which can mate to form the *a*/ α diploid. The mating reaction is initiated by peptide pheromones secreted by each haploid cell type. α cells secrete the pheromone α -factor which acts on *a* cells, and *a* cells secrete *a*-factor which acts on α cells. Pheromones binding to specific receptors on target cells activate a signaling pathway, ultimately leading to transcriptional changes, alteration of cellular morphology ('shmoo' formation) and arrest of mitotic cell division in G₁. However, the mating pheromone response is transient because cells adapt to the continued presence of pheromone and resume the mitotic cycle (for review, see Marsh *et al.*, 1991).

Genetic analysis of the pheromone response pathway has led to the identification of many components required for signal transduction. The *STE2* and *STE3* genes encode the receptors for α -factor and *a*-factor, respectively, and their primary structures are predicted to contain seven

transmembrane domains, a characteristic of mammalian G protein-coupled receptors (Burkholder and Hartwell, 1985; Nakayama *et al.*, 1985; Hagen *et al.*, 1986). *GPA1*, *STE4* and *STE18* encode the α , β and γ subunits of a G protein that is thought to be functionally coupled to the pheromone receptor (Dietzel and Kurjan, 1987; Miyajima *et al.*, 1987; Jahng *et al.*, 1988; Blinder *et al.*, 1989; Whiteway *et al.*, 1989). In the current model for signal transmission, pheromone binding to the receptor promotes the exchange of GDP for GTP, resulting in dissociation of the G $\beta\gamma$ complex from the GTP-bound G α subunit. Free G $\beta\gamma$ complex then propagates the signal by an unknown mechanism involving *STE5* to a group of protein kinases encoded by the *STE20*, *STE7*, *STE11*, *FUS3* and *KSS1* genes (Leberer *et al.*, 1993; for review, see Marsh *et al.*, 1991).

The pheromone response pathway kinases are thought to function in the order *STE11*, *STE7*, *FUS3*/*KSS1* (Cairns *et al.*, 1992; Gartner *et al.*, 1992; Stevenson *et al.*, 1992; Errede *et al.*, 1993; Zhou *et al.*, 1993). Both *FUS3* and *KSS1* belong to the mitogen-activated protein (MAP) kinase family, which plays a key role in a variety of signal transduction pathways (Courchesne *et al.*, 1989; Elion *et al.*, 1990, 1991). *STE7* is most closely related to the MAP kinase activator (Crews *et al.*, 1992) and is able to phosphorylate and activate *FUS3* *in vitro* (Errede *et al.*, 1993).

Ultimately, activation of the MAP kinase family members leads to transcriptional activation of mating-specific genes and to cell cycle arrest. The transcriptional effects are mediated by the *STE12* transcription factor (Dolan *et al.*, 1989; Errede and Ammerer, 1989). Cell cycle arrest by mating pheromone appears to occur by inactivation of three functionally redundant G₁-specific cyclins (*CLN1*, *CLN2* and *CLN3*) required for G₁ function of the *CDC28* protein kinase (Wittenberg *et al.*, 1990). One of the physiological targets of *FUS3* is *FAR1*, which is a pheromone-inducible phosphoprotein that mediates inactivation of *CLN2* and *CLN1* (Chang and Herskowitz, 1990; Peter *et al.*, 1993; Valdivieso *et al.*, 1993).

The responsiveness of a signal transduction system to a persistent stimulus diminishes with time. This phenomenon, known as desensitization or adaptation, is a universal characteristic of signal response systems. Several molecular mechanisms for desensitization have been described in the pheromone response pathway. Mutations in the *SST2* gene confer defects in adaptation and increased pheromone sensitivity (Chan and Otte, 1982), indicating that *SST2* is required for adaptation to pheromone although its function is unknown. The hyperactivating mutation *GPA1*^{Val50}, which is analogous to the oncogenic *ras*^{Val12} mutation, has a dominant hyperadaptive phenotype and is capable of suppressing the adaptation defect of *ssr2* mutants (Miyajima *et al.*, 1989). Thus it is thought that the activated G α subunit stimulates an adaptive response antagonistic to the signal generated by the G $\beta\gamma$ complex. Additionally, *GPA1* and *SST2* lie on different adaptive pathways.

To elucidate further the components operating in the adaptive response, we isolated genes which when overexpressed from multicopy plasmids are capable of suppressing the lethality of *gpa1*⁻ mutants, which is due to the constitutive activity of the free G β γ subunits. We anticipated that overexpression of components downstream of GPA1 in the adaptation pathway would stimulate recovery from growth arrest. One such gene, which we have named *MSG5*, is described here. It encodes a putative protein tyrosine phosphatase. We analyzed the phenotypes resulting from overexpression and disruption of *MSG5* with respect to pheromone response. In addition, we deduced the point at which *MSG5* functions in the pheromone response pathway. Based on these results, we discuss the possible role of the *MSG5* protein in the recovery pathway.

Results

Isolation of high copy number plasmids which suppress the *gpa1*⁻ mutation

We isolated high copy number plasmids containing *S.cerevisiae* genomic DNA, that allowed *gpa1*⁻ cells to grow under restrictive conditions. For this purpose, a yeast strain (KMG59) with a chromosomal deletion of the *GPA1* gene was constructed that contained the *GPA1* gene under the control of the *GAL1* promoter on a plasmid (pG1501). As a functional *GPA1* gene is required for haploid cell growth, this strain grows in medium containing galactose, where the *GAL1* promoter is induced. In medium containing glucose, the *GAL1* promoter is repressed. Under these conditions, GPA1 is depleted and the cells arrest in the G₁ phase of the cell cycle (Miyajima *et al.*, 1987).

The conditional *gpa1*⁻ strain (KMG59 pG1501) was transformed with a yeast genomic library constructed in the multiple copy vector YEp213, which contains the yeast *LEU2* gene for selection. Leu⁺ transformants were screened by plating directly on glucose-based medium to select for suppression of the *gpa1*⁻ phenotype. Eight colonies were dependent on a library plasmid for their ability to grow on glucose. Each isolate grew on glucose-based medium even upon loss of the *GAL1*–*GPA1* plasmid (pG1501). The latter result excludes the possibility that any of the genes suppressed the *gpa1*⁻ phenotype by allowing the *GAL1* promoter to work in the presence of glucose. The

suppressing plasmids were recovered in *Escherichia coli* and shown to rescue the *gpa1*⁻ phenotype when reintroduced into KMG59 cells. The DNAs from these plasmids could be grouped into five classes by restriction analysis. We named these genes *MSG* for multicopy suppressor of *gpa1*, and the *MSG5* gene was characterized in this study.

Effect of *MSG5* on mating pheromone signal transduction

The ability of *MSG5*-overexpressing cells to respond to mating pheromone was studied. Mating pheromone induces transcription of mating-specific genes and causes G₁ arrest. One test for normal transcriptional induction is the level of STE12-dependent transcription. When analyzed using a reporter plasmid (pUZ4) carrying a pheromone-inducible *lacZ* gene, *MSG5* overexpression caused ~50% inhibition of the α -factor-inducible β -galactosidase activity (Table I). Halo bioassays were performed to examine the growth arrest response to pheromone induction. Wild-type cells containing *MSG5* on a high copy number plasmid recovered more quickly from pheromone-induced growth arrest than did cells with the control plasmid (Figure 1A). To examine further the recovery promoted by *MSG5* overexpression, experiments were performed in an *sst2* mutant background. The *SST2* gene product is involved in recovery from pheromone-induced arrest and *sst2* mutants are more sensitive to pheromone than wild-type cells (Chan and Otte, 1982). *MATa sst2* cells transformed with YEpMSG5 displayed an apparent sensitivity to pheromone quantitatively similar to the same cells transformed with the control plasmid (YEp13). However, the halos produced with *MSG5*-overexpressing lawns subsequently became turbid and eventually filled in (Figure 1B). Thus, *MSG5* overexpression caused cells to overcome pheromone-induced arrest and resume growth, suggesting that *MSG5* is a component of the recovery pathway.

Because overexpression of *MSG5* dramatically promoted resuming growth of pheromone-arrested cells, we tested the effect of a *MSG5* disruption on cell growth and pheromone response. The chromosomal *MSG5* gene of a wild-type diploid strain 15D (*leu2/leu2*) was transplaced by a DNA fragment containing *MSG5* with a *LEU2* selectable marker inserted within part of the coding region (Figure 2). Upon sporulation and tetrad dissection, all four spores were viable

Table I. Effect of *MSG5* on transcription from a pheromone-inducible promoter

Strain	Plasmid	β -galactosidase activity			
		– α -factor	+ α -factor	Glucose	Galactose
KMG60-4C	YEp13	3.1	45.6	–	–
	YEpMSG5	2.4	26.6	–	–
KMG60-3D	YEp13	28.4	67.1	–	–
	YEpMSG5	8.7	38.0	–	–
15Dau-GS	YEp13	0.6	23.6	0.6	53.8
	YEpMSG5	0.9	9.3	0.5	55.2

KMG60-4C (*MATa STE11*⁺) and KMG60-3D (*MATa STE11*⁻⁴) contained the reporter plasmid pUZ4. Cells were grown in synthetic medium containing the appropriate amino acids and 2% glucose, and were induced with 2 μ g/ml α -factor for 3 h. 15Dau-GS (*MATa*) contained plasmids pGS3 (pGAL-STE12) and pUZ4. Cells were grown in synthetic medium containing the appropriate amino acids and 2% glucose, and were induced with 2 μ g/ml α -factor for 3 h. For induction of *STE12* cells were shifted from sucrose medium to glucose or galactose medium, and were grown for an additional 6 h. The units shown are the average of two or three experiments in which two independent Leu⁺ plasmid-bearing transformants were assayed.

and *msg5-1::LEU2* spore clones exhibited no obvious growth defect compared with isogenic wild-type spore clones. Therefore, *MSG5* is not required for vegetative growth. *MATa msg5-1::LEU2* strains were slightly more sensitive to α -factor-induced cell cycle arrest than isogenic wild-type strains by halo bioassays (data not shown). An *MSG5* deletion mutation in which 71% of coding sequence is replaced by the *LEU2* gene (*msg5 Δ -2::LEU2*) was also constructed and tested (Figure 2). This deletion mutation has the same phenotype as the disruption allele (*msg5-1::LEU2*). Compared with the pheromone sensitivity of *sst2⁻* cells, that of *msg5⁻* cells is much weaker. The minimal effect of *msg5* on the pheromone sensitivity may reflect the presence of a functionally redundant gene or other mechanisms of adaptation may remain available to *msg5⁻* cells.

A *GPA1^{Val50}* mutation causes hyperadaptation to α -factor (Miyajima *et al.*, 1989). This mutation is believed to increase the activity of the $G\alpha$ subunit by decreasing its intrinsic GTPase activity. Thus *GPA1* appears to play a positive role in the transduction of signals which stimulate recovery from pheromone-induced growth arrest. If *MSG5* is a target for this *GPA1* signal, loss of the *MSG5* (*msg5-1::LEU2*) should block the ability of *GPA1^{Val50}* to promote recovery. Since *GPA1^{Val50}* is dominant with respect to *GPA1⁺* for adaptation (Miyajima *et al.*, 1989), the plasmid pG1606

carrying *GPA1^{Val50}* was transformed into *MATa* wild-type and *msg5-1::LEU2* strains and the ability of cells to respond to mating pheromone was assayed (Figure 1C). As observed previously (Miyajima *et al.*, 1989), *GPA1^{Val50}* in wild-type cells markedly accelerated recovery from pheromone response. In contrast, *GPA1^{Val50}* was unable to promote adaptation in *msg5-1::LEU2* cells. These results suggest that *MSG5* functions downstream of *GPA1* in the pathway and that *MSG5* is a potential transducer of the $G\alpha$ subunit signal.

***MSG5* shares homology with the active site of protein tyrosine phosphatases**

Plasmid YEpMSG5 causes three distinct phenotypes: suppression of *gpa1⁻* deletion, promotion of recovery from pheromone-induced growth arrest and a decrease in transcription of pheromone response genes activated by α -factor. Subcloning experiments showed that a single segment (2 kb *HindIII*) of YEpMSG5 was responsible for all three phenotypes (Figure 2), suggesting that overexpression of a single gene (*MSG5*) promotes these activities. The nucleotide sequence of this essential region and surrounding regions was determined. Sequence analysis of the *MSG5* gene revealed an open reading frame (ORF) of 489 amino acid residues (Figure 3). This size is consistent with the length

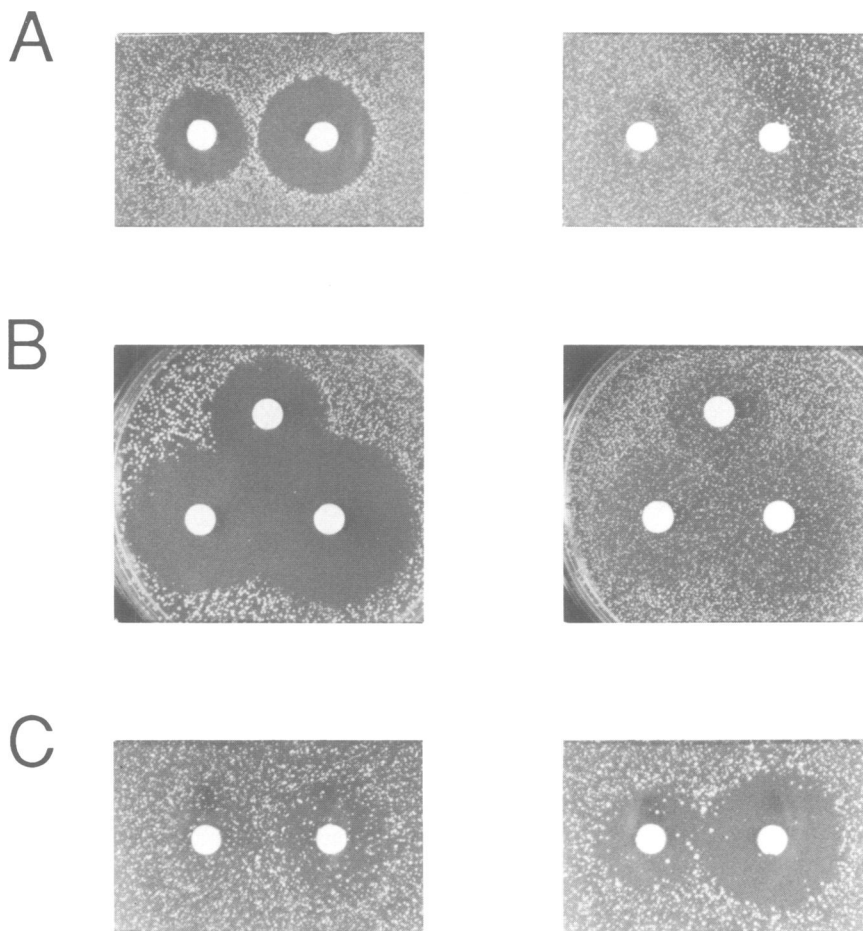


Fig. 1. Response to α -factor. Cells were plated on soft agar. Sterile filter disks were placed on the nascent lawn and samples of synthesized α -factor (Sigma) were pipetted onto the disks. Plates were incubated at 30°C for 2 days. The amount of α -factor added to each disk was: (A and C) 1.6 μ g (left) and 8 μ g (right); (B) 0.32 μ g (top), 1.6 μ g (left) and 8 μ g (right); (A) 15Dau (wild-type) with YEp181 (left) or YEpMSG5 (right); (B) BC180 (*sst2⁻*) with YEp13 (left) or YEpMSG5 (right); (C) DD1-2B (*MSG5⁺*) with pG1606 (*GPA1^{Val50}*) (left), DD1-2D (*msg5-1::LEU2*) with pG1606 (right).

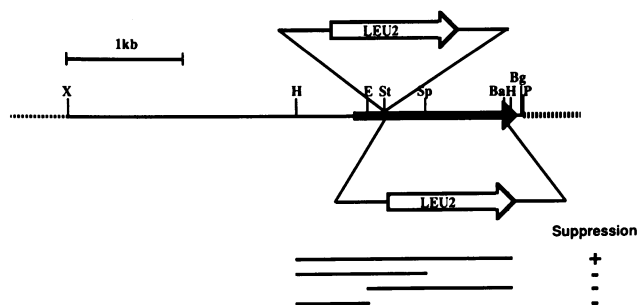


Fig. 2. Restriction map and disruption of *MSG5*. The top line represents an abbreviated restriction map of the *MSG5* region. The arrow represents the ORF and direction of transcription of *MSG5* (see Figure 6). Restriction sites: Ba, *BalI*; Bg, *BglII*; E, *EcoRI*; H, *HindIII*; P, *PstI*; Sp, *SphI*; St, *SnaI*; X, *XbaI*.

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1  MQFHSDKQHL  DSKTDIDFKP  NSPRSLQNRN  TKNLSLDIAA
41  LHPIMEFSSP  SQDVPGSVKF  PSPTPLNLFM  KPKPIVLEKC
81  PPKVSPRPTP  PSLSMRRSEA  SIYTLPTSLK  NRTVSPSVYT
121 KSTVSSISK  LSSSSPLSSF  SEKPHLNRVH  SLSVTKDLK
161 LKGIRGRSQT  ISGLETSTPI  SSTREGTLDL  TDVNRFSNQK
201 NMQTTLIFPE  ESDDLNIDMV  HAEIYQRTVY  LDGPLLVLP
241 NLYLYSEPKL  EDILSFDLVI  NVAKEIPNLE  FLIPPEMAHK
281 IKYYHIEWTH  TSKIVKDLR  LTRIIHTAHS  QGKKILVHCQ
321 CGVSRSSALI  VAYIMRYGL  SLNDAYDELK  GVAKDISPNM
361 GLIFQLMEWG  TMLSKNSPGE  EGETVHMPEE  DDIGNEVSS
401 TTKSYSSASF  RSFFPMVTNLS  SSPNDSSVNS  SEVTPRTPAT
441 LTGARTALAT  ERGEDDEHCK  SLSQPADSLE  ASVDNESIST
481 APEQMMFLP

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Fig. 3. Predicted amino acid sequence of the *MSG5* gene product. Nucleotide sequence was determined as described in Materials and methods. The GenBank accession number is D17548. The HC motif is boxed.

MSG5	GKKILVHCQCGVSRSSALIVAYIMRYGLSLND--
PAC-1	GGRVLVHCQAGISRSATICLAYLMQSRRLVLE--
CL100	GGRVFLVHCQAGISRSATICLAYLMRTNRVKLDE--
VHR	NGRVLVHCREGYSRPTLVIAVLMRQKMDVKS--
YVH1	RGAVFAHCQACLRSVTFIVAYLMRYGLSLM--
VH1	NEPVLVHCAAGVNRSGAMILAYLMSKNKESSPMLY

MSG5	---AYDELKGVAKDISPNMGLIFQLMEWGCTMLSKN
PAC-1	---AFDFVKQRRGVISPNFSFMGQLLOFETQVLCH
CL100	---AFEFVKQRRSIIISPNSFMGQLLOFESQVLAP
VHR	---ALSVIRQNR-EIGPNDGFLAQLCOLNDRLAKE
YVH1	---AMHAVKRRKPSVEPNENFMQLHLFEKMGDF
VH1	FLYVYHSMRDLRGAFVENPSFKRQIEE-KYVIDKN

Fig. 4. Amino acid sequence alignment of *MSG5*, *PAC-1*, *CL100*, *VHR*, *YVH1* and *VH1*. The conserved amino acid residues are boxed. Asterisks indicate the residues that are completely identical and they are shaded. Dashes are inserted to allow for maximal alignment of the sequences.

of the transcript (~1.8 kb) identified by hybridization analysis of total RNA (see Figure 6).

The predicted protein sequence was compared with those in the protein structural motif database Prosite (Bairoch, 1991). The search revealed that the *MSG5* polypeptide contains a sequence related to an 'HC motif' (Ile/Val-His-CysXXXXXArg) that comprises the conserved active site of protein tyrosine phosphatases (PTPases) (Fisher *et al.*, 1991). The C-terminal portion of the *MSG5* protein has significant amino acid sequence similarity to a phosphatase encoded by the late H1 gene of vaccinia virus (*VH1*), which is able to dephosphorylate protein substrates modified on both tyrosine and serine residues (Figure 4) (Guan *et al.*, 1991).

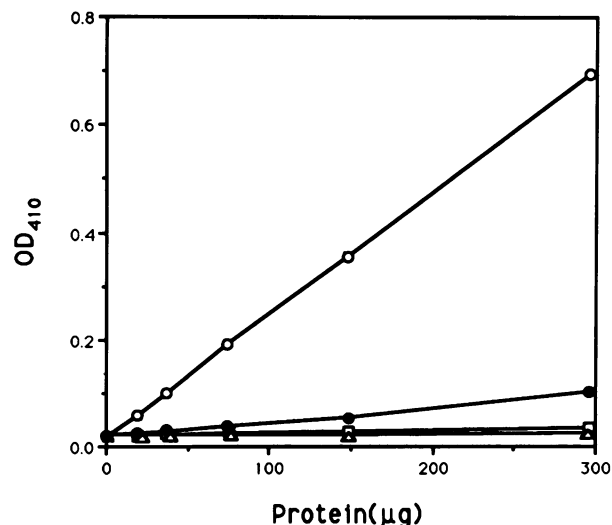


Fig. 5. Phosphatase activity. GST-*MSG5* (○), GST-*MSG5*^{Ala319} (□) and GST (△) were expressed in bacteria and purified in parallel by using glutathione-Sepharese CL-4B. Dephosphorylation of pNPP, expressed as increased absorbance at 410 nm, was measured as a function of protein concentration. The GST-*MSG5* fusion protein was also incubated in the presence of 1 mM sodium vanadate (●).

Members of a family of PTPases with similarity to *VH1* are now expanding and include a growth factor-inducible gene in murine 3T3 cells (*3CH134*) (Charles *et al.*, 1992) and its human homolog (*CL100*) (Keyse and Emslie, 1992), a mitogen-inducible gene in human T cells (*PAC-1*) (Rohan *et al.*, 1993), the *VH1*-related proteins in human fibroblasts (*VHR*) and yeast (*YVH1*) (Guan *et al.*, 1992; Ishibashi *et al.*, 1992) (Figure 4). *VHR* has been shown to be a dual specificity phosphatase which dephosphorylates both serine and tyrosine residues *in vitro* (Ishibashi *et al.*, 1992). This group of phosphatases most likely represents a new structural family of PTPases that share regulatory or enzymatic properties.

To determine whether the *MSG5* protein possesses phosphatase activity, the complete ORF of *MSG5* was cloned into a bacterial expression vector to generate a fusion gene encoding a glutathione *S*-transferase fragment (GST) followed by *MSG5*. Soluble GST-*MSG5* protein was readily produced at high levels in bacteria. This fusion protein was purified by glutathione affinity chromatography. GST-*MSG5* was found to hydrolyze rapidly *p*-nitrophenyl phosphate (pNPP), a chromogenic substrate structurally related to phosphotyrosine (Figure 5). This phosphatase activity was inhibited by sodium vanadate, a well-known inhibitor of PTPases.

The cysteine residue in the HC motif is absolutely essential for phosphatase activity (Streuli *et al.*, 1990; Guan *et al.*, 1991). To confirm that the cysteine present in the *MSG5* HC motif is essential for phosphatase activity, we performed site-directed *in vitro* mutagenesis to change the *MSG5* codon for Cys319 to Ala319 (see Materials and methods). GST-*MSG5*^{Ala319} was produced in bacteria in an identical manner to wild-type GST-*MSG5* and tested in parallel for its ability to dephosphorylate pNPP. As expected for a catalytically inactive derivative, the GST-*MSG5*^{Ala319} protein did not hydrolyze pNPP (Figure 5). We also examined the *MSG5*^{Ala319} mutation *in vivo* for its ability to affect pheromone-induced signal transduction. The

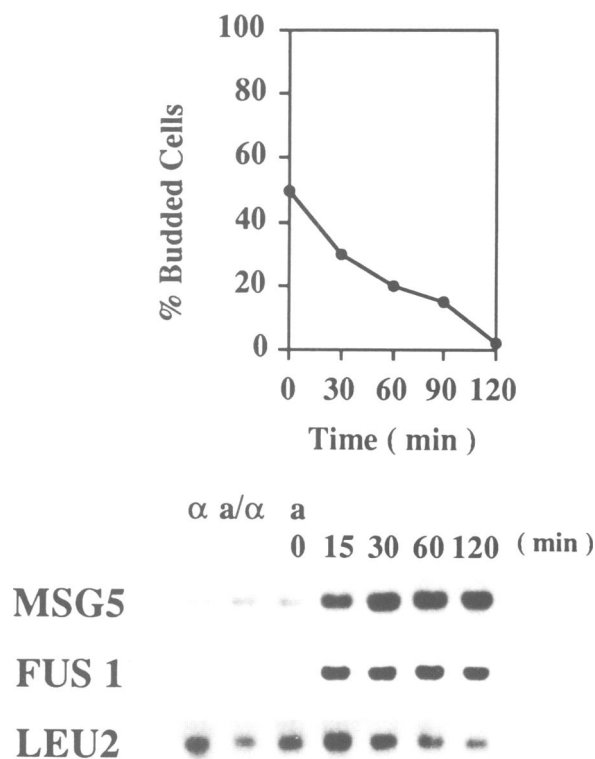


Fig. 6. Northern analysis of *MSG5* transcription. Three micrograms of total RNA prepared from 15D α (*MAT α*), 15D (*MATa/MAT α*) and 15Dau-b (*MATa bar1⁻*) were run on 1% formaldehyde-agarose gels and analyzed by Northern blot analysis using *MSG5* and *FUS1* probes. The same blots were analyzed using a *LEU2* probe as a control. To analyze the *MSG5* transcript in cells responding to α -factor, samples were taken from a culture of 15Dau-b at the indicated times following addition of α -factor (40 ng/ml). Samples were analyzed for the proportion of budded cells (top panel) and Northern blot analysis. The intensity of *MSG5* bands was quantitated by densitometric tracing with a film of a lesser exposure.

MSG5^{Ala319} mutation lost the ability to promote recovery from pheromone-induced cell cycle arrest even when the mutated form of *MSG5* was overexpressed under the control of *GAL1* promoter (data not shown).

Regulation of *MSG5* gene expression

Northern analysis with an *MSG5* probe revealed a single major message of ~1.8 kb. This transcript was observed in haploid cells of both mating types as well as in *MATa/MAT α* diploids (Figure 6). To see whether α -factor could modulate *MSG5* expression, an asynchronous culture was treated with α -factor and analyzed for the presence of *MSG5* transcripts at the designated intervals over the next 2 h (Figure 6). The top panel shows that asynchronous cells treated in this way arrested within a single cell cycle as unbudded G₁ cells. The *MSG5* transcript increased ~5-fold in response to mating pheromone treatment. This accumulation is maximal within 30 min following addition of α -factor. Thus the kinetics of this increase appear to be slower than the transcriptional response of the pheromone-induced *FUS1* gene, which reaches its plateau within 15 min after α -factor addition (McCaffrey *et al.*, 1987).

The increase of the *MSG5* transcript in response to α -factor could be either a direct result of pheromone action or an indirect affect mediated by synchronization at the G₁

Table II. List of strains

Strain	Genotype
15D	<i>MATa/MATα ade1/ade1 his2/his2 leu2/leu2 trp1/trp1 ura3/ura3</i>
15D α	<i>MATα ade1 his2 leu2 trp1 ura3</i>
15Dau	<i>MATa ade1 his2 leu2 trp1 ura3</i>
15Dau-b	<i>MATa bar1 ade1 his2 leu2 trp1 ura3</i>
15Dau-GS	<i>MATa ade1 his2 leu2 trp1 ura3</i> [pGS3 (pGAL-STE12)]
BC180	<i>MATa sst2-Δ2 ade2 his3 leu2 ura3</i>
DD1-2B	<i>MATa ade1 his2 leu2 trp1 ura3</i>
DD1-2D	<i>MATa msg5-1::LEU2 ade1 his2 leu2 trp1 ura3</i>
KMG59	<i>MATa gpa1::HIS3 his3 leu2 trp1 ura3</i> [pG1501 (pGAL1-GPA1)]
KMG60-3D	<i>MATa STE11-4 his3 leu2 trp1 ura3</i>
KMG60-4C	<i>MATa his3 leu2 trp1 ura3</i>

interval of the cell cycle. The *MSG5* transcript levels were characterized by Northern blot analysis of total RNA prepared from samples taken at 20 min intervals after release from the mating pheromone-induced G₁ arrest. The accumulation of the *MSG5* transcript decreased following removal of α -factor and *MSG5* mRNA failed to accumulate prior to the second cycle of bud emergence corresponding to the G₁ interval (data not shown). These results indicate that the accumulation of the *MSG5* transcript in response to α -factor is not just the consequence of synchronization in G₁.

We tested whether transcriptional induction of *MSG5* is sufficient for activation of *MSG5* in the pheromone response. To obtain conditional, high level expression of the *MSG5* gene in yeast cells, the *MSG5* ORF was fused to the *GAL1* promoter. In the presence of galactose, wild-type cells carrying a plasmid with *GAL1-MSG5* did not respond to α -factor for growth arrest and they were mating defective (data not shown).

Mapping *MSG5* function in the pheromone response pathway

The phenotype of a strain that combines mutations in genes affecting two different steps in a cellular process can provide information about the order in which two gene products function. To learn where *MSG5* functions with respect to other signaling components in the pathway, we examined the effect of *MSG5* overexpression on constitutive activation by a hyperactive allele of *STE11* (*STE11-4*) (Stevenson *et al.*, 1992) or by overproduction of the *STE12* gene product (Dolan and Fields, 1990). A plasmid carrying a pheromone-inducible *lacZ* gene (pUZ4) was used to monitor transcriptional activation by measurement of β -galactosidase activity. When the reporter plasmid was present in wild-type *MATa* cells, α -factor stimulated the production of β -galactosidase by >10-fold (Table I). Overexpression of *MSG5* caused an ~2-fold decrease in expression of *lacZ* compared with α -factor stimulated wild-type cells. The *STE11-4* mutation or overexpression of the wild-type *STE12* gene by the galactose-inducible *GAL1* led to constitutive transcription of the reporter gene, as judged by the increased level of β -galactosidase activity in the absence of α -factor (Table I). Overexpression of *MSG5* resulted in a decrease in the constitutive activity by the *STE11-4* mutations, whereas it did not affect the constitutive level in the case of *STE12* overproduction. Assuming a linear transmission pathway for

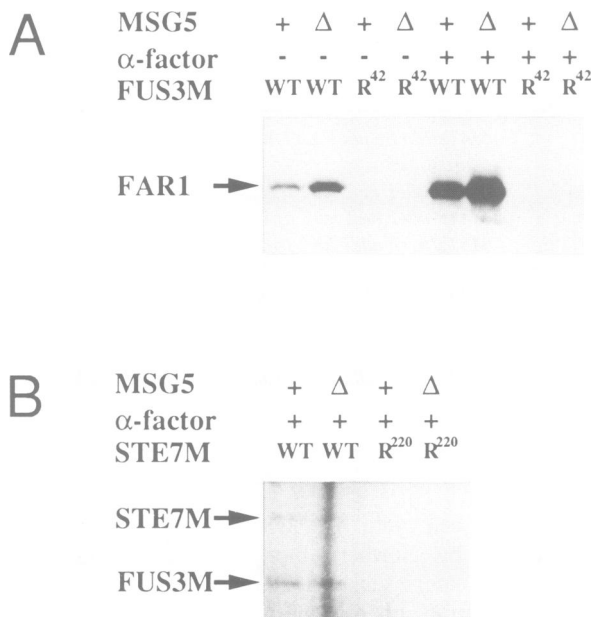


Fig. 7. *In vitro* FUS3 and STE7 kinase activities. (A) Comparison of kinase activity for FUS3 isolated from *msg5*⁻ disruption strain (DD1-2D, Δ) or *MSG5*⁺ strain (DD1-2B, +). Uninduced cultures or pheromone-induced cultures (4 μM α-factor, 1 h) of these strains were the source protein extract for FUS3M (wt) or FUS3M^{Arg42} (R⁴²) immune complexes that are present in reactions as indicated. The target substrate was an N-terminal fragment of FAR1 (250 ng) and was present in all assays. (B) Comparison of kinase activity for STE7 isolated from *msg5*⁻ disruption strain (DD1-2D, Δ) or *MSG5*⁺ strain (DD1-2B, +). Pheromone-induced cultures (4 μM α-factor, 1 h) of these strains were the source protein extract for STE7M (wt) or STE7M^{Arg220} (R²²⁰) immune complexes that are present in reactions as indicated. The target substrate, FUS3M^{Arg42} (250 ng), was present in all assays.

the signal, these results position the action of MSG5 after the STE11 protein kinase but prior to the STE12 transcription factor in the pathway.

Three protein kinases, STE7, FUS3 and KSS1, have been implicated as components acting between STE11 and STE12 in the pheromone response pathway (Marsh *et al.*, 1991). After pheromone treatment, FUS3 and KSS1 rapidly become phosphorylated at tyrosine and threonine residues. For FUS3 it has been shown that phosphorylation at both residues is essential for function (Gartner *et al.*, 1992). Because MSG5 has the structural and enzymatic characteristics of protein tyrosine phosphatases, one attractive hypothesis for its role in promoting adaptation is that MSG5 dephosphorylates and, thereby, inactivates FUS3 (and KSS1).

According to the above hypothesis, loss of MSG5 function should enhance FUS3 kinase activity. To test this possibility, we performed immune complex phosphorylation assays with a myc epitope tagged version of FUS3 (FUS3M) isolated from *msg5-1::LEU2* disruption cells (DD1-2D) and from cells that express MSG5 (DD1-2B). We used an N-terminal fragment of the FAR1 protein as the FUS3-specific substrate (Errede *et al.*, 1993; Peter *et al.*, 1993). A stronger signal for FAR1 phosphorylation resulted when FUS3M was isolated from pheromone-induced cells which lack MSG5 than from cells which express MSG5 (Figure 7A, compare lanes 5 and 6). Although the overall amount of FAR1 phosphorylation was less, a similar effect of *msg5* disruption was observed when FUS3M was isolated from uninduced cells (Figure 7A, lanes 1 and 2). The absence of FAR1

phosphorylation in parallel reactions using the catalytically inactive FUS3M^{Arg42} substitution derivative confirms that FUS3M is responsible for the FAR1 phosphorylation observed in these assays (Figure 7A, lanes 3, 4, 7 and 8). Because the FUS3M protein for these experiments was expressed from the constitutive *TP11* promoter, its expression was independent of the genetic background or pheromone-induced state. Additionally, immune blot analysis confirmed that the same amount of protein was present in extracts used for immune complex formation (data not shown). These results are consistent with FUS3 being in a more active state when it is isolated from cells that do not express MSG5.

To distinguish whether the effect of the *msg5* disruption is specific for FUS3 or might generally affect kinase activities, we performed analogous immune complex phosphorylation assays with a myc epitope tagged version of STE7 (STE7M). For these assays, we used the catalytically inactive FUS3M^{Arg42} which had been isolated from *E. coli* as the STE7-specific substrate (Errede *et al.*, 1993). In contrast to the results with FUS3, disruption of *msg5* did not result in increased amounts of STE7M-catalyzed phosphorylation (Figure 7B, lanes 1 and 2). This result excludes the possibility that *msg5* disruption has a general affect on protein kinase activities. Instead, these results raise the possibility that the MSG5 promotes cellular adaptation to pheromone response by inhibiting FUS3.

MSG5 inactivates FUS3 *in vitro*

To test directly whether MSG5 is a protein phosphatase with specificity for phosphorylated FUS3, we examined the effect of MSG5 on the phosphorylation state and activity of autophosphorylated FUS3. MSG5 and FUS3 were expressed as GST fusion proteins in *E. coli* and these soluble proteins were purified by affinity chromatography. The activity of FUS3 was assayed by its ability to phosphorylate a truncated FAR1 protein that was also produced and purified from *E. coli* (Peter *et al.*, 1993). Thus, all proteins for this test were isolated from a background lacking potential contaminating kinases or phosphatases.

Autophosphorylation of bacterially produced FUS3 leads predominantly to the modification of Tyr182 and an unidentified serine residue (Errede *et al.*, 1993). When the FUS3 autophosphorylation time was extended, we found some phosphorylation also occurring at Thr180 (Figure 8D). The specific activity of such preparations also appeared to be higher than that of the previously reported autophosphorylation product (data not shown). We added GST-MSG5 or GST-MSG5^{Ala319} to the high activity form of autoactivated FUS3. After the indicated incubation times, we then measured kinase activity with FAR1 protein as substrate (Figure 8A, lanes 2–4 and 6–8). Treatment of FUS3 with GST-MSG5 for increasing time resulted in a progressive decrease in FUS3 phosphorylation and a concomitant loss of kinase activity (Figure 8A, lanes 2–4). Phosphoamino acid analysis of FUS3 indicated that the abundance of all three phosphoamino acids is reduced by the GST-MSG5 treatment (Figure 8B, compare lanes 13 and 14, panels D and E). The mutant form of the protein phosphatase seems to retain some activity, although it is much less pronounced compared with the wild-type product (Figure 8A, lanes 1–8). Control experiments showed that addition of GST polypeptide alone has no effect on the phosphorylation state of FUS3 and that GST-MSG5 does not directly dephosphorylate FAR1 (data not shown).

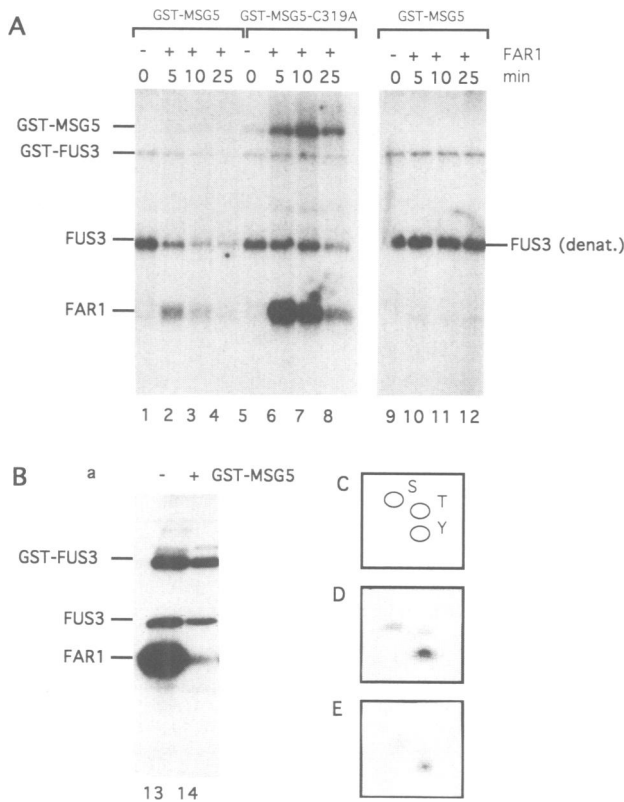


Fig. 8. Autophosphorylated FUS3 is dephosphorylated and inactivated by MSG5. (A) Autoradiogram of kinase activity assays. *In vitro* phosphorylated FUS3 was treated with GST-MSG5 (lanes 1–4 and 9–12) or GST-MSG5^{Ala319} (lanes 5–8). Incubation times were as indicated. FAR1 was added (+) or not (–) after phosphatase treatment. In lanes 9–12, phosphorylated FUS3 was heat denatured before addition of GST-MSG5. The positions of GST-MSG5, GST-FUS3, FUS3 and FAR1 are indicated. (B) shows an experiment similar to panel A with FUS3 kinase activity before (lane 13) and after GST-MSG5 treatment (lane 14). (C–E) Phosphoamino acid analysis of autophosphorylated FUS3. (C) The positions of phospho-amino acid standards are indicated. S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine. (D and E) Phosphorimager prints presenting the phosphoamino acid analyses of FUS3 cut out from lanes 13 and 14, respectively.

Together these results suggest that phosphatase activity of MSG5 is necessary for the observed inactivation of FUS3. Because FUS3 enzyme activity is completely inhibited while some phosphorylated product remains, we cannot rule out that steric mechanisms may also contribute to the inhibition. For example, MSG5 may compete with FAR1 for the enzyme active site.

Two additional observations are noteworthy. First, we found that a native conformation of FUS3 is important for its dephosphorylation by MSG5. When autophosphorylated FUS3 was denatured by elevated temperature (5 min at 90°C) prior to the addition of GST-MSG5, there was no decrease in FUS3 phosphorylation (Figure 8A, lanes 9–12). This finding may also explain why in some experiments loss of kinase activity preceded complete dephosphorylation of FUS3 (for example, see Figure 8B, lanes 13 and 14). This dependence on a native conformation is consistent with MSG5 having a stringent substrate specificity. Second, in addition to FAR1, the GST-MSG5 product also becomes phosphorylated in these reactions. This effect is most apparent using the GST-MSG5^{Ala319} substitution mutant that does not inactivate FUS3 (Figure 8A, lanes 6–8). Thus,

MSG5 is a potential target for FUS3 although it remains to be demonstrated that FUS3-dependent phosphorylation occurs *in vivo* and is of functional significance.

Discussion

MSG5 has a role in adaptation to pheromone

Desensitization or adaptation is a property of most eukaryotic signal transduction systems. Adaptive processes may function at several levels within the signal transduction system, including that of the receptor and downstream transducing components. Often these processes require induction by the stimulating agent and therefore constitute a specific response to the signal. When yeast cells are exposed to prolonged treatment with mating pheromone, they undergo an adaptive response and resume growth (Moore, 1984). Thus pheromone-dependent signal transduction in *S.cerevisiae* provides an opportunity to investigate the interactions between a signal transduction pathway and its adaptive mechanisms.

We described the cloning and characterization of a novel gene, *MSG5*, which accelerates recovery from pheromone when overexpressed. For several reasons we believe that *MSG5* is part of an adaptive system as opposed to interfering nonspecifically with the function of a signaling component. First, loss of *MSG5* function reduces the ability to adapt. This phenotype is most apparent in connection with the *GPA1*^{Val50} allele, a mutation which normally allows cells to recover faster from G₁ arrest. Second, transcription of the gene is significantly induced after pheromone exposure. In this regard *MSG5* behaves similarly to *SST2*, another gene which plays an essential role in recovery. *MSG5*, therefore, satisfies the criteria that adaptive functions should be induced as part of the overall response.

Origin of the adaptive signal

Unlike most other eukaryotic G protein-dependent signaling systems, activation of the signal transduction system is not carried out by the G α subunit but by the G $\beta\gamma$ dimer (for review, see Marsh *et al.*, 1991). However, there is evidence suggesting that the dissociated G α subunit encoded by *GPA1* is important for adaptation (Miyajima *et al.*, 1989; Stone and Reed, 1990). A *GPA1*^{Val50} mutation which is analogous to a constitutively activated mutation in *ras* (*ras*^{Val12}) causes accelerated recovery from pheromone. Since such a mutation should increase the level of GTP bound G α by decreasing its intrinsic GTPase activity, it has been proposed that the activated G α stimulates an adaptive response that is transduced separately from the mating signal generated by G $\beta\gamma$ (Miyajima *et al.*, 1989). The discovery of the *MSG5* gene has provided an opportunity to test this hypothesis further. Our genetic analysis showed that the effect of the *GPA1*^{Val50} allele is dependent on a functional *MSG5* gene, indicating that *MSG5* is a potential target for a G α -mediated signal. *GPA1* might induce a post-translational modification of *MSG5* that results in enhanced protein phosphatase activity. Alternatively, *GPA1* might be involved in the transcriptional induction of *MSG5*.

Previously, the *SGV1* product was identified as a potential component of a G α signaling pathway (Irie *et al.*, 1991). The *SGV1* gene is essential for growth and encodes a kinase with homology to CDC28. At the permissive temperature *sgv1-1* mutants are supersensitive to pheromone and suppress hyperadaptation in *GPA1*^{Val50} strains (Irie *et al.*, 1991).

Overexpression of *MSG5*, however, was unable to suppress either the growth defect or supersensitivity of the *sgv1* mutation (K. Irie and K. Matsumoto, unpublished observations). Assuming that both gene products function in the same adaptive pathway, either *MSG5* acts prior to *SGV1* or its activity is dependent on *SGV1*.

How does *MSG5* promote adaptation to pheromone?

The nucleotide sequence of the *MSG5* gene strongly suggests that it is a protein with PTPase activity. The most striking similarity occurs in the region of a conserved cysteine, the so-called HC motif, which is believed to form a critical part of the active site of PTPases. Like other PTPases, bacterially produced *MSG5* protein was effective at dephosphorylating *p*-nitrophenyl phosphate. This hydrolytic activity was inhibited by orthovanadate. Furthermore, a mutation changing codon Cys319 to Ala319 inactivated the enzyme *in vitro* and abolished the ability of *MSG5 in vivo* to stimulate the adaptation to pheromone. Thus, the *MSG5* protein displays the properties that are hallmarks of known PTPases.

Since *FUS3* and *KSS1* are the only known components of the signaling pathway whose activity requires tyrosine phosphorylation, they were prime candidates for targets of *MSG5*. This prediction was confirmed by the demonstration that a GST-*MSG5* fusion protein dephosphorylates and inactivates *in vitro* phosphorylated *FUS3*. Genetic analyses are also consistent with the view that *MSG5* antagonizes the signal pathway at the position of *FUS3*. Epistatic interactions imply that *MSG5* functions between *STE11* and *STE12*. Furthermore, disruption of the *MSG5* gene enhanced *FUS3*-dependent kinase activity, whereas activity of *STE7* remained unaffected. In preliminary experiments we found that overexpression of *MSG5* suppresses pheromone-induced modification of *FUS3* (A. Gartner, unpublished observations). Taken together these data suggest that *FUS3* is at least one of the physiological targets of *MSG5*. It is also likely that dephosphorylation of *FUS3* is the major mechanism by which *MSG5* desensitizes the $G\beta\gamma$ -dependent signal transduction system.

Our data suggest that *MSG5* belongs to a novel subclass of protein phosphatases whose substrates are MAP kinase family members. Our observations showed that *MSG5* is highly discriminatory for its protein substrate, while exhibiting multiple specificities for the modified amino acid. Interestingly, a related phosphatase from mammalian cells (CL100) has recently been described and also found to dephosphorylate both the phosphotyrosine and phosphothreonine of recombinant MAP kinase *in vitro* (Alessi *et al.*, 1993). Therefore, MAP kinases in general may be regulated by a dual specificity threonine/tyrosine kinase and a dual specificity threonine/tyrosine phosphatase. *MSG5* will become a very useful tool for dissecting the opposing interactions which establish the level of MAP kinase activity.

Materials and methods

Yeast strains, media and genetic manipulation

The *S. cerevisiae* strains used in this study are listed in Table II. The compositions of rich and synthetic media, supplemented with the appropriate nutrients for plasmid maintenance, have been described previously (Miyajima *et al.*, 1989; Nomoto *et al.*, 1990). Standard genetic procedures for yeast were used (Sherman *et al.*, 1986). Yeast transformations were carried out by the alkali cation method (Ito *et al.*, 1983).

Plasmids

The plasmid pG1606 is YCpN1 (*TRP1* as a selection marker) containing the *GPA1*^{Val50} gene (Miyajima *et al.*, 1989). Plasmids pG33 (Dolan and Fields, 1990) and pG1501 (Miyajima *et al.*, 1987) carry the *STE12* and *GPA1* structural genes, respectively, fused with the *GAL1* promoter. These are YCp-based plasmids with *URA3* as a selection marker. The reporter plasmid pUZ4 (*TRP1* as a selection marker in YCp-based plasmid) contains three consensus binding sites for the transcriptional factor *STE12* upstream of the β -galactosidase gene. Details of the construction and structure of this plasmid are described by Cairns *et al.* (1992). YCpG33 is a centromere-based *URA3* plasmid containing the *GAL1* promoter (K. Sugimoto, unpublished). Plasmids pNC318, pNC318-R220, pGA1903 and pGA1905 that were used for expression of *STE7M*, *STE7M*^{Arg220}, *FUS3M* and *FUS3M*^{Arg42}, respectively, have been described (Gartner *et al.*, 1992; Zhou *et al.*, 1993).

Cloning of *MSG5* and gene disruptions

The *MSG5* gene was cloned from the YEpl3-based genomic DNA library (provided by Akio Sugino) as the plasmid, YEplMSG5. This clone had an insert of ~10 kb. Plasmid YEplMSG5 was constructed by inserting 4 kb *XbaI*–*PstI* fragment of YEplMSG5 into the vector YEplac181.

Two disruption mutations of the *MSG5* gene were constructed as follows. pSP contained the 2 kb *HindIII* fragment of the *MSG5* gene from YEplMSG5 cloned in pSP73 (Promega). To make the first disruption (*msg5-1::LEU2*), pSPdis was created by inserting *LEU2* into the *SauI* site of *MSG5* (Figure 2), located 100 amino acids from the N-terminus of the putative protein. A *PvuII*–*SphI* fragment of pSPdis containing the *msg5-1::LEU2* construct was used to transform a diploid strain 15D by selection for *Leu*⁺. Southern analysis of genomic DNA from the resulting transformants was conducted to confirm that transplacement had occurred at the *MSG5* locus. The transformant, heterozygous at the *MSG5* locus (*MSG5/msg5-1::LEU2*), was designated DD1. To make the second *MSG5* disruption (*msg5Δ-2::LEU2*), the 1.4 kb *EcoRI*–*PstI* fragment of the *MSG5* gene from YEplMSG5 was cloned in pSP73. pSPdel was constructed by replacing the internal *SauI*–*BalI* fragment (Figure 2) with the *LEU2* gene. A *BglIII* fragment of pSPdel containing the *msg5Δ-2::LEU2* construct was used to transform 15D. Southern hybridization and tetrad analysis confirmed that the transformed diploids carried a single copy of the *msg5Δ-2::LEU2* construction.

Sequencing of *MSG5*

Restriction endonuclease fragments of the 2 kb *HindIII* and 1.4 kb *EcoRI*–*PstI* were subcloned into pSP72 and pSP73. The sequences of both strands were determined using Sequenase (US Biochemical Corp.). The 3'-region of *MSG5* overlapped with an ORF residing 5'-side of the *COX5* gene (Seraphin *et al.*, 1985).

Construction of YCpGMSG5

The *MSG5* coding sequence was amplified by a PCR using a 5' primer (CTCGGATCCATGCAATTTCACTCAG) and a 3' primer (CTCGGATCCTTAAGGAAGAAAC) both incorporating a *BamHI* site. PCR amplification generated a 1.5 kb fragment that was digested with *BamHI* and inserted into the *BamHI* site of YCpG33 to generate YCpGMSG5.

Expression of *MSG5* in bacteria

In order to express *MSG5* in *E. coli*, the *BamHI* fragment of YCpGMSG5 was subcloned into the *BamHI* site of pGEX-KG (Guan and Dixon, 1991) to produce pGEX-MSG5. JM109 *E. coli* transformed with pGEX-MSG5 were grown in L-broth at 37°C to an optical density of 0.5 and induced for an additional 3 h in the presence of 1 mM IPTG. GST-*MSG5* fusion protein was purified as previously described (Millar *et al.*, 1991).

Site-directed mutagenesis

A mutation of *MSG5* (*MSG5*^{Ala319}) containing alanine instead of cysteine at a position 319 was prepared as follows. The first PCR was performed with SP6 primer (Takara) as the 5' primer and the 3' primer GATCTTGATACTCCACACTGCGCATGTACGAGTATTTCTTG containing an *FspI* site. The second PCR was performed with the 5' primer GGCAAGAAAATACTCGTACATGCGCAGTGTGGACTATCAAGATC containing an *FspI* site and T7 primer (Stratagene) as the 3' primer. Both PCRs used pSP2 as the template. The first PCR fragment was digested with *EcoRI* and *FspI* and the second PCR fragment was digested with *FspI* and *PstI*. These two digested fragments were ligated into *EcoRI*–*PstI* site of pSP73 to obtain pSPmut. The DNA sequence between *SphI* and *PstI* sites on pSPmut was confirmed. A 0.9 kb *SphI*–*XhoI* fragment of pGEX-MSG5 was replaced with the DNA fragment from pSPmut containing the mutation to produce pGEX-MSG5^{Ala319}. YCpGMSG5^{Ala319} was constructed by inserting the *BamHI*–*XhoI* fragment of pGEX-MSG5^{Ala319} into the *BamHI*–*SalI* site of YCpG33.

Northern blotting

Northern blot analysis was performed as described previously (Wittenberg *et al.*, 1990). The DNA fragments used as probes are as follows: *MSG5*, the 1.4 kb *EcoRI*–*PstI* fragment from the coding region of *MSG5*; *FUS1*, the 1.4 kb *EcoRI* fragment from plasmid pBS231 (Trueheart *et al.*, 1987); *LEU2*, the 1.42 kb *Clal*–*XhoI* fragment from plasmid YEp13.

Cell cycle synchronization by mating pheromone

Synchronization by mating pheromone was accomplished as described previously (Wittenberg *et al.*, 1990).

Pheromone sensitivity assays

A halo bioassay was used to measure the sensitivity of cells to α -factor-induced growth arrest. After growth to mid-log phase, $\sim 10^5$ cells were diluted into 8 ml of soft agar and spread onto plates. Disks of chromatography paper (6 mm diameter; Whatman) were placed on the nascent lawn. Different concentrations of synthetic α -factor (Sigma) were dotted onto the disks in 4 μ l aliquots.

β -galactosidase assays

β -galactosidase assays were performed as described previously (Miyajima *et al.*, 1989).

Phosphatase assays

Dephosphorylation of pNPP was performed as described previously (Millar *et al.*, 1991).

In vitro FUS3 and STE7 kinase assays

The FAR1 N-terminal fragment used as the specific substrate for FUS3 kinase assays was expressed in *E. coli* and isolated as previously described (Peter *et al.*, 1993). The FUS3M^{Arg42} protein used as the specific substrate for STE7 kinase assays was expressed in *E. coli* and isolated as previously described (Errede *et al.*, 1993). Conditions for immune complex isolation of FUS3M and STE7M and for phosphorylation assays were as previously described (Errede *et al.*, 1993), with the exception that cold ATP was included in the FUS3M kinase reaction at a concentration of 100 μ M.

Protein phosphatase assays

Bacterially produced GST–FUS3 product was purified and cleaved to obtain FUS3 as described by Errede *et al.* (1993). The 251 amino acid N-terminal fragment of FAR1 was produced as described by Peter *et al.* (1993). GST–MSG5 was isolated as described above and was stored after elution from glutathione beads in 50% (vol) glycerol, 10 mM DTT, 10 mM MgCl₂ and 1 mM MnCl₂. FUS3 was phosphorylated at 30°C for 3.5 h after combining 67 μ l reaction buffer (25 mM MOPS pH 7.2, 15 mM MgCl₂, 5 mM EGTA, 10 mM DTT and 1 mM MnCl₂), 10 μ l 1 mM ATP and 5.5 μ l [γ -³²P]ATP (55 μ Ci), 15 μ l FUS3 (3 μ g) and 5.2 μ l 250 mM MOPS pH 7.2. After 3.5 h, 90 μ l reaction buffer was added and the mixture was divided into 35 μ l aliquots. Six microlitres of GST–MSG5 or GST–MGSS^{Ala319} (~ 60 ng) were added to each sample. For studies with denatured FUS3, the reaction mix was heated to 90°C for 5 min and cooled before adding phosphatase. After 0, 5, 10 and 25 min of incubation at 30°C the reactions were either stopped immediately by adding 15 μ l of SDS sample buffer or after 1 μ l of FAR1 (1 μ g) was added for an additional 5 min. Proteins were separated by SDS–PAGE and visualized by autoradiography. Labeled FUS3 was cut out of the dried gel and subjected to phosphoamino acid analysis as described (Cooper *et al.*, 1983).

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